The N terminus of the Drosophila Numb protein directs membrane association and actin-dependent asymmetric localization

JUERGEN A. KNOBLICH*, LILY Y. JAN, AND YUH NUNG JAN

Departments of Physiology and Biochemistry, Howard Hughes Medical Institute, University of California, San Francisco, CA 94143-0724

Contributed by Yuh Nung Jan, September 24, 1997

ABSTRACT Drosophila Numb is a membrane associated protein of 557 amino acids (aa) that localizes asymmetrically into a cortical crescent in mitotic neural precursor cells and segregates into one of the daughter cells, where it is required for correct cell fate specification. We demonstrate here that asymmetric localization but not membrane localization of Numb in Drosophila embryos is inhibited by latrunculin A, an inhibitor of actin assembly. We also show that deletion of either the first 41 aa or aa 41–118 of Numb eliminates both localization to the cell membrane and asymmetric localization during mitosis, whereas C-terminal deletions or deletions of central portions of Numb do not affect its subcellular localization. Fusion of the first 76 or the first 119 aa of Numb to β-galactosidase results in a fusion protein that localizes to the cell membrane, but fails to localize asymmetrically during mitosis. In contrast, a fusion protein containing the first 227 aa of Numb and β-galactosidase localizes asymmetrically during mitosis and segregates into the same daughter cell as the endogenous Numb protein, demonstrating that the first 227 aa of the Numb protein are sufficient for asymmetric localization.

Asymmetric cell divisions in which a mother cell segregates a determinant into one of its daughter cells to make this cell different from its sister cell contribute to generating different cell types in a multicellular organism (1, 2). In Drosophila, the four cells that form an external sensory (ES) organ—one type of sensory structure found in the peripheral nervous system—arise during development from a single sensory organ precursor (SOP) cell that undergoes a series of asymmetric cell divisions (3–5). During these asymmetric divisions, the protein Numb acts as a segregating determinant. In wild-type animals, the SOP cell divides into a IIA cell and a IIB cell. The IIA cell gives rise to the two outer cells (hair and socket cell) whereas the IIB cell forms the two inner cells (neuron and sheath cell). In a numb mutant, the SOP cell divides into two IIA cells that give rise to four outer cells and no inner cells (6). Conversely, when the numb gene is overexpressed, the SOP cell divides into two IIB cells that form four inner cells and no outer cells (7). numb is required for correct cell fate specification not only during SOP cell division, but also during the division of the IIA and IIB cells (6–8). numb encodes a membrane associated protein that contains an N-terminal phosphotyrosine binding domain (9). In interphase cells, the protein is uniformly distributed around the circumference of the cell. In mitotic SOP cells, however, Numb concentrates in the membrane area that overlies one of the two centrosomes and preferentially enters one of the two daughter cells (7, 10). Several experiments indicate that Numb acts by inhibiting signaling via the Notch transmembrane receptor in this daughter cell (11–13).

Asymmetric localization of Numb is not restricted to SOP cells. During stage 9 of Drosophila embryonic development, epithelial cells in the procephalic neurogenic region of the ectoderm divide perpendicularly to the epithelial surface (14) and each give rise to a large apical daughter cell and a smaller basal daughter cell. Numb is preferentially segregated into the basal daughter cell during this division (15), but the function of Numb in this case has not been characterized. Neuroblasts, the progenitor cells of the central nervous system, each divide into a large apical daughter cell that retains neuroblast characteristics and a smaller basal ganglion mother cell (GMC) that later divides into two neurons (16). Numb is segregated into the GMC during neuroblast division. In the absence of Numb, however, GMCs are not transformed into additional neuroblasts, even though defects in some central nervous system lineages are observed in numb mutants (17).

Like Numb, the Drosophila protein Prospero localizes asymmetrically in dividing neuroblasts and SOP cells. Prospero is a nuclear transcription factor, which is required for correct cell fate specification in the central nervous system and for neuronal differentiation in the peripheral nervous system (18, 19). During mitosis, the protein transiently translocates to the cell membrane and colocalizes with Numb (10, 20, 21). Numb and Prospero enter the same daughter cell, where Numb stays at the membrane whereas Prospero enters the nucleus. Numb and Prospero localization are independent events (10, 21), but localization of both proteins requires the protein Insoluble. Insoluble also localizes asymmetrically in dividing neuroblasts, but it is localized to the side opposite to that of Numb and Prospero localization prior to Numb and Prospero asymmetric localization (15, 22). In insoluble mutants, Numb and Prospero either do not localize asymmetrically or form crescents at random positions around the cell (15). Insoluble is also involved in the orientation of the mitotic spindle. In insoluble mutants, the mitotic spindle in neuroblasts is oriented randomly, and ectopic expression of insoluble in epithelial cells causes a reorientation of their mitotic spindle (15). Thus, Insoluble directs and coordinates several processes during asymmetric cell division.

Localization of Numb, Prospero, and Insoluble does not require microtubules, but disruption of actin filaments causes defects in asymmetric localization (10, 15). After treatment with cytochalasin D, Insoluble is no longer asymmetrically localized (15). Numb and Prospero still localize asymmetrically, but their crescents are frequently misoriented (10). We show here that treatment with latrunculin A, a more potent inhibitor of actin polymerization, completely abolishes Numb and Prospero asymmetric localization, suggesting that crescent

Abbreviations: β-gal, β-galactosidase; ES, organ external sensory organ; PTB, phosphotyrosine binding; SOP, sensory organ precursor; GMC, ganglion mother cell.

*Present address: Research Institute of Molecular Pathology, A-1030 Vienna, Austria.
formation requires actin filaments. We also demonstrate that a small region at the N terminus of the Numb protein is required for both membrane association and asymmetric localization. When fused to β-gal, this region is sufficient for membrane localization, but not for asymmetric localization. In contrast, a β-gal fusion protein containing a much larger N-terminal fragment that includes the PTB domain is asymmetrically localized in the dividing precursor cell and then segregated into one of the two daughter cells.

**METHODS**

**numb Deletion and numb-lacZ Fusion Constructs.** The plasmid pSKnb-myc and the deletion constructs numb-ΔI, numb-ΔJ, numb-ΔK, numb-ΔL, and numb-ΔPTB have been described before (11). For RNA injection, a vector was used that contains an SP6 promoter and β-globin 5'[3' untranslated sequences (described in ref. 23, will be called pXBG below). For cloning into this vector, an NcoI site was introduced at the translation initiation site of pSKnb-myc by PCR, yielding pSKnb-myc-NcoI. The deletion constructs were cloned into pSKnb-myc-NcoI using unique restriction sites flanking the deletions. The complete ORF was then inserted into pXBG using NcoI and XbaI generating pXBG-nb-myc-NcoI and derivatives.

numb-Δmyr was generated in a PCR reaction that changed the second codon of the numb coding region from GGA to GCC and introduced an NcoI site at the translation start. numb-ΔN was generated in a PCR reaction that introduced an NcoI site at the second ATG codon (nucleotides 915–917 of the published cDNA, ref. 6). The PCR fragments were cloned into pSKnb-myc-NcoI containing NcoI and SphI and the resulting cDNAs were inserted into pXBG using NcoI and XbaI.

numb-04-lacZ was generated by replacing a BamHI/XbaI fragment of pXBG-nb-myc-NcoI containing the Numb C terminus (from nucleotide 1472) with a XmaI/SpeI fragment containing the lacZ gene from pPDF8.02 (24). The other numb-lacZ fusions were generated by PCR from pSK-numb-myc-NcoI using primers that introduced BamHI sites after nucleotides 914 (numb-01-lacZ), 1019 (numb-02-lacZ), or 1145 (numb-03-lacZ). PCR fragments were cloned into numb-04-lacZ via NcoI/BamHI.

To generate transgenic flies, numb-ΔN was cloned into pUAST (25) using EcoRI/XbaI. The numb-lacZ fusion constructs were cloned into pSKnumb-myc-NcoI via NcoI/StuI, excised with NotI and cloned into pUAST. Transgenic flies were generated using a w; Oregon-R stock following standard protocols.

**RNA Injection Experiments.** Capped RNA was generated from the various pXBG-constructs using the SP6 Message-Machine kit (Ambion) and injected into stage 4 Drosophila embryos. The embryos were aged to stage 9 at 18°C, transferred to test tubes with heptane, fixed, and stained for immunofluorescence.

**Immunofluorescence and Confocal Microscopy.** Drosophila embryos were fixed and processed for immunofluorescence essentially as described before (7), except that 5% paraffomaldehyde was used as fixative and 2% normal donkey serum was used as blocking reagent. Rabbit anti-Numb (7), rabbit anti-Prospero (19), and rabbit anti-β-gal (Cappel) were used as primary antibodies. DTAF donkey-anti-rabbit (The Jackson Laboratory) was used as secondary antibody, and propidum iodide (26) was used to stain DNA. Embryos were mounted in Slow Fade mounting medium (Molecular Probes) and analyzed on a Bio-Rad MRC 600 confocal microscope.

**Drug Treatment Experiments.** For treatment with latrunculin A, Drosophila embryos at the desired stage were dechorionated for 3 min with 50% household bleach and transferred to test tubes in embryo wash buffer (0.7% NaCl/0.07% Triton-X100). They were overlayed with a 1:1 mixture of n-heptane and Drosophila Schneider’s medium (GIBCO) containing latrunculin A (Molecular Probes) dissolved in dimethyl sulfoxide or just dimethyl sulfoxide as a control. After incubation with gentle shaking at room temperature for the desired time, the Schneider’s medium was replaced with 5% paraformaldehyde and the embryos were fixed for 20 min. Fifty percent of the embryos were devitellinized with methanol and used for antibody staining according to standard protocols (7). The rest of the embryos were devitellinized by hand and stained with rhodamine-phalloidin (Molecular Probes, 1:50) for 15 min.

**RESULTS**

**Latrunculin A Disrupts Asymmetric Localization of Numb and Prospero.** Disrupting the actin cytoskeleton with cytchalasin D does not abolish the asymmetric localization of Numb and Prospero (10) even in cells where the drug treatment leads to a defect in cytokinesis. Recent experiments have shown that the drug latrunculin A is more potent than cytchalasin D in disrupting the actin cytoskeleton (27, 28). We have tested the effect of latrunculin A on the asymmetric localization of Numb and Prospero (Fig. 1). Rhodamine-phalloidin staining reveals that treatment of Drosophila embryos with 200 μM latrunculin A for 20 min results in almost complete depolymerization of F-actin (Fig. 1B), though some very faint residual staining at the cell cortex can still be detected at higher amplification using the confocal microscope (data not shown). Both Numb and Prospero are no longer asymmetrically localized but remain membrane associated in embryos treated with latrunculin A.

![Fig. 1. Latrunculin A inhibits asymmetric localization of Numb and Prospero.](image-url)
culin A (Fig. 1 D and F), whereas their localization is not affected in embryos processed in parallel but not exposed to the drug (Fig. 1 C and E). Even when the drug concentration is increased to 400 μM and the treatment extended to 40 min, Numb and Prospero remain associated with the cell membrane (data not shown). We conclude that the asymmetric localization of Numb and Prospero is dependent on actin filaments.

The Numb N Terminus, but Not the N-Myristoylation Signal, Is Required for Asymmetric Localization. To identify domains in the Numb protein that are essential for membrane localization and asymmetric localization, we generated a series of deletion constructs (Fig. 2) and analyzed the subcellular localization of these mutant Numb proteins in embryos injected with RNA generated from these deletion constructs. This analysis is limited to early stages of Drosophila development, because the mRNA has to be injected before cellularization and will be degraded with time. We therefore analyzed the asymmetric localization of the modified Numb proteins in the cells of the procephalic neurogenic region of the ectoderm, the first embryonic cells that localize Numb asymmetrically. Stage 4 embryos were injected with capped mRNA, then aged to stage 9 of development, fixed and analyzed by immunofluorescence using an anti-Numb antibody (7) (Fig. 3). The tagged wild-type Numb-myc and the mutant proteins with C-terminal or central deletions (Numb-D2, Numb-D3, and Numb-D4) exhibited the same distribution as the endogenous Numb protein (Fig. 3 A and D–G). The proteins were found at the cell membrane in all cell types, and in the cells of the procephalic neurogenic region, they segregated into the basal daughter cell during mitosis. In contrast, the two most N-terminal deletions (Numb-ΔN, Numb-Δ1) caused reproducible abnormalities in localization (Fig. 3 B and C). In all cell types, the proteins with N-terminal deletions were found mostly in the cytoplasm and failed to segregate preferentially into one of the two daughter cells during mitosis. We conclude that the N terminus of the Numb protein is required for both asymmetric localization and localization to the cell membrane.

The N terminus of the Numb protein contains a consensus site for N-myristoylation (6). To test whether this site is required for localizing the Numb protein to the cell membrane, we substituted the glycine at position 2 of the Numb protein with alanine, a mutation that has been shown to completely abolish N-myristoylation in other proteins (29). No defects in membrane association and asymmetric localization were found when mRNA from this mutant construct (numb-Δmry) was injected into embryos (Fig. 3H). Whether Numb is N-myristoylated in vivo is not known. If N-myristoylation of Numb does take place, it does not appear to be required for association with the cell membrane or for asymmetric localization (see Discussion).

Different Requirements for Asymmetric Localization and Downstream Function of Numb in Overexpression Experiments. The overexpression of Numb in SOP cells leads to a cell fate transformation of the IIA cell into a second IIB cell, resulting in ES organs with four inner cells and no outer cells (7). Occasionally, only the second cell division is affected, resulting in ES organs with two hairs, but no socket. To identify the regions of Numb necessary for this overexpression function, we generated transgenic flies carrying several of the Numb deletion constructs. The results obtained for Numb-Δ2 and Numb-ΔPTB have been described (11). Consistent with our RNA injection results, no defects in asymmetric localization and membrane localization were observed when these constructs were expressed in mitotic neuroblasts (11). The same results were obtained when the localization of Numb-Δ2 and Numb-ΔPTB was analyzed in numb1 protein null-mutant embryos (data not shown). When overexpressed in SOP cells of adult ES organs, however, both proteins were completely nonfunctional (11).

Transgenic flies were also generated for numb-ΔN and the UAS/GAL4 system was used to express numb-ΔN under the control of the hairy promoter (25) in both epidermal cells and

![Fig. 3. Asymmetric segregation of Numb deletion constructs in RNA injection experiments. Stage 4 Drosophila embryos were injected with capped mRNA generated from Numb deletion constructs, aged to stage 9, then fixed and stained with an anti-Numb antibody (green) and propidium-iodide (DNA, red). Optical cross sections (apical up, basal down) through the procephalic neurogenic region of stage 9 embryos are shown. Numb-myc (A), Numb-Δ2 (D), Numb-Δ3 (E), Numb-Δ4 (F), Numb-ΔPTB (G), and Numb-Δmry (H) are localized to the cell membrane and segregate normally into the basal daughter cell (arrow), whereas Numb-ΔN (B) and Numb-Δ1 (C) are localized in the cytoplasm and segregate equally into both daughter cells.](http://www.pnas.org/content/94/13/6742/fig/3)

![Fig. 2. Map of the Numb deletion and Numb-β-gal fusion constructs used in this study. The numbers refer to aa positions in the published sequence (6).](http://www.pnas.org/content/94/13/6742/fig/2)
The N-Terminal Half of the Numb Protein Is Sufficient for Asymmetric Localization. Asymmetric localization and membrane localization of Numb require the N terminus. To test whether the N terminus of the Numb protein is sufficient for asymmetric localization, we generated constructs encoding N-terminal fragments of Numb fused with the Escherichia coli β-gal protein (Fig. 2). numb-01-lacZ contains the first 41 aa, numb-02-lacZ the first 76 aa, numb-03-lacZ the first 119 aa, and numb-04-lacZ the first 227 aa of Numb. We generated transgenic flies carrying these fusion constructs and used the UAS/GAL4 system to express the constructs under the control of the scabrous promoter (25). The subcellular localization of the fusion proteins was analyzed in neuroblasts of stage 10 embryos by immunofluorescence using an anti-β-gal antibody and propidium iodide to stain DNA (Fig. 5). Numb-01-β-gal was found in the cytoplasm with no signs of asymmetric localization during mitosis (Fig. 5A). Numb-02-β-gal and Numb-03-β-gal were localized to the cell membrane, but failed to localize asymmetrically in mitotic neuroblasts. The proteins remained uniformly distributed around the cell cortex instead and segregated into both daughter cells (Fig. 5B and C). In contrast, Numb-04-β-gal was localized to the cell membrane in interphase cells, became localized asymmetrically in mitotic neuroblasts and then segregated into the ganglion mother cell (Fig. 5D). The same results were obtained when the localization of the Numb-β-gal fusion proteins was analyzed in numb1 protein null mutant embryos (data not shown). We conclude that the first 76 aa of Numb are sufficient to direct membrane localization but not asymmetric localization of β-gal, while the first 227 aa of Numb can direct asymmetric localization and segregation of β-gal preferentially into one of the two daughter cells.

DISCUSSION

During mitosis of Drosophila neural precursor cells, the proteins Numb and Prospero localize asymmetrically and segregate into one of the two daughter cells. We show here that asymmetric localization of Numb and Prospero, but not localization to the cell membrane, can be eliminated by the drug latrunculin A that disrupts the actin cytoskeleton. Furthermore, we demonstrate that the first 119 aa of Numb are essential for asymmetric localization and for localization to the cell membrane, while most of the central and C-terminal portions of Numb are not required. When the first 119 or the first 76 aa of Numb are fused to β-gal, however, they can direct localization to the cell membrane but not asymmetric localization. In contrast, a larger N-terminal fragment containing the first 227 aa, which include the PTB domain,
is sufficient for asymmetric localization and segregation into one daughter cell.

**Numb and Prospero Localization Are Actin Dependent Processes.** In many organisms, asymmetric segregation of determinants during mitosis is thought to be mediated by actin-dependent mechanisms. In *Caenorhabditis elegans*, the first cell division of the zygote is asymmetric and establishment of asymmetry during this division requires actin but not microtubules (30, 31). In *Saccharomyces cerevisiae*, ASH1 mRNA segregates into one of the two daughter cells during mitosis (32, 33), where it is translated to generate the transcriptional repressor Ash1p (34, 35). The process of Ash1 segregation requires a functional actin cytoskeleton, but not microtubules (32).

In *Drosophila*, the asymmetric segregation of Numb and Prospero during mitosis has been shown to be microtubule independent (10) and experiments using the actin drug cytochalasin D have suggested that actin is also not required for Numb and Prospero localization. After cytochalasin D treatment, Numb and Prospero are still asymmetrically localized in cells that have defects in other actin dependent processes, such as cytokinesis (10). However, the crescents frequently form at incorrect positions and are no longer strictly correlated with the position of one of the two spindle poles. We have investigated the actin dependence of Numb and Prospero localization using the drug latrunculin A (28, 36), which has recently become available. In tissue culture cells, latrunculin A has been shown to be a more potent actin inhibitor than cytochalasin D (28). Indeed, we find that after treatment of *Drosophila* embryos with latrunculin A, actin filaments are almost completely undetectable by rhodamine-phalloidin staining (Fig. 1B), whereas actin filaments are still present (though severely disorganized) after treatment with cytochalasin D (10). Furthermore, in contrast to cytochalasin D treatment, treatment with latrunculin A completely inhibits the asymmetric localization of Numb and Prospero. It is unlikely that our observations are due to nonspecific effects of the drug because the effects of latrunculin A at concentrations similar to the ones used in our experiments in yeast can be completely suppressed by several point mutations in the actin gene that all map to the same putative binding site (27).

Despite its effect on asymmetric localization, latrunculin A fails to disrupt membrane localization of Numb and Prospero, suggesting that these two proteins are anchored to the cell membrane by an actin independent process. However, we could reproducibly detect some residual rhodamine-phalloidin staining at the cell cortex after latrunculin A treatment of *Drosophila* embryos, even when the drug concentration was increased to 400 μM (data not shown). This could be due to nonspecific rhodamine-phalloidin staining or reflect an incomplete disassembly of the actin cytoskeleton. Latrunculin A inhibits the polymerization of actin, but does not actively depolymerize actin filaments (27) and cortical actin filaments in *Drosophila* cells could have a low turnover rate that makes them resistant to latrunculin A. Thus, we cannot exclude that membrane localization of Numb and Prospero is mediated by actin filaments that are resistant to the drug treatment.

**Domains that Direct Membrane Localization and Asymmetric Localization of Numb.** Our experiments show that the first 119 aa of the Numb protein contain a region that is both necessary and sufficient for localization to the cell membrane. Deletions of the first 42 aa or of aa 40–119 result in a cytoplasmic protein that is no longer asymmetrically localized (Fig. 3B and C), whereas a fusion protein between the first 119 aa of Numb and β-gal is localized to the cell membrane. The N terminus of Numb contains an N-myristoylation signal, but mutation of this signal sequence in Numb-Dμn does not disrupt membrane localization (Fig. 3H). Numb-Dμn could be localized to the cell membrane by associating with endogenous Numb protein. Alternatively, Numb could be localized to the cell membrane by a process that does not require the N-myristoylation signal. The Numb N terminus is not predicted to form a transmembrane domain, but interaction of the N terminus with an integral or peripheral membrane protein or phospholipid interactions are alternative possibilities that could account for membrane localization of Numb.

Deletion analysis of the Numb protein has identified a 118 aa region that directs localization to the cell membrane and asymmetric localization of β-gal fusion proteins (20). A 13-aa motif within this region has some weak homology to aa 379–391 in Numb (20) and it has been suggested that this region might be involved in asymmetric localization of both proteins. However, Numb-D4 that removes these 13 aa localizes asymmetrically and segregates into one daughter cell (Fig. 3F) suggesting that this region is not required for asymmetric localization. The observation that the first 227, but not the first 119 aa of Numb are sufficient for asymmetric localization might indicate that aa 119–227 contain the localization domain. However, Numb-DΔ that deletes aa 117–216 and Numb-DPTB that deletes aa 79–203 still localize asymmetrically even in the absence of endogenous Numb protein. Several possibilities remain to be tested: It could be that the localization domain lies between aa 217 and 227. Alternatively, asymmetric localization could be mediated by two partially redundant domains. One of these domains would be located within aa 119–227, but in Numb-DΔ and Numb-DPTB a second, more C-terminal domain could mediate asymmetric localization. In any case, the finding that the first 227 aa of Numb are involved in asymmetric localization suggests that proteins that bind to the N-terminal half of Numb may be involved in its asymmetric localization.

We thank Yee-Ming Chan, Salim Abdelilah, and Liqun Luo for helpful comments on the manuscript and all the members of the Jan Lab for fruitful discussions. We also thank Katja Brose for valuable help with generating the Numb deletion constructs. J.A.K. was supported by an European Molecular Biology Organization postdoctoral fellowship and by the Howard Hughes Medical Institute. L.Y.J. and Y.N.J. are Howard Hughes Investigators.